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Relationship between degradation of wood and production of H_2O_2-producing or one-electron oxidases by brown-rot fungi

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Summary. The one-electron oxidation activity of brown-rot fungi was determined by measuring ethylene production from KTBA. Ethylene production was related to degradation of lignin, cellulose, and wood itself. Extracellular protein that catalyzed oxidation of KTBA was isolated from wood-containing cultures. This protein required H_2O_2 for KTBA oxidation. It was also found to oxidize NADH, producing H_2O_2 via O_2^- in the presence of O_2. The protein showed little phenol-oxidase activity under conditions giving high activity against KTBA. The results indicate that partially reduced oxygen may play a role in the initial degradation of the cellulose and lignin in wood in cultures of brown-rot fungi.

Introduction

Cellulose breakdown in wood by fungi is generally thought to be a result of the combined action of several extracellular hydrolytic enzymes (Labavitch 1981). Many cellulases capable of depolymerizing cellulose have been isolated from white-rot and deuteromycetous fungi. However, attempts to isolate extracellular hydrolytic enzymes that are able to depolymerise microcrystalline cellulose from brown-rot fungi have been unsuccessful.

When brown-rot fungi were cultured on pure cellulose or lignin-related compounds, most of them failed to degrade the substrates (Highley 1973; Nilsson 1974; Enoki et al. 1985). However, when growing on wood, most of the brown-rot fungi break through the lignin barrier and degrade the cellulose in preference to the lignin (Kirk, Highley 1973; Enoki et al. 1985). Recently it has been suggested that brown-rot fungi may generate a unique wood-component-degrading system that can split cellulose as well as lignin into fragments. This suggestion is based on the facts that brown-rot fungi degrade cellulose only under conditions in which the ligninolytic system of the fungi is also produced, and the cellulolytic system is necessarily produced by brown-rot fungi under conditions in which ligninolytic system of the fungi is produced (Enoki et al. 1988).

Polysaccharides can be oxidized and depolymerized by hydroxyl radical (Halliwell 1965; Gilbert et al. 1984). The pattern of progressive change in the average degree of polymerization of the holocellulose in wood induced by the Fenton's reagent is very similar to that of wood attacked by brown-rot fungi, but not to that of white-rot fungi

(Cowling 1961; Halliwell 1965). Thus alternative mechanisms of degradation have been proposed involving small non-enzyme degrading agents such as H_2O_2-Fe^{++} system (Koenigs 1974). It has been found that hydrogen peroxide solution (1 mM) in the absence of metallic catalysts and potassium superoxide can depolymerize cell polysaccharides including cellulose (Thompson, Corbett 1985; Miller 1986).

The facts mentioned above suggest that hydrogen peroxide may be involved directly or indirectly in initial breakdown of the cellulose or the lignin in wood by brown-rot fungi. For the H_2O_2 system to be operative, an oxidative enzyme must be produced that generates hydrogen peroxide by oxidation of electron donors. The pyridine nucleotides NADH and NADPH are known to be involved in many biological H_2O_2-generating systems. Peroxidases that reduce O_2 and generate H_2O_2 with NADH or NADPH are widely distributed in living organisms. Peroxidases have been intensively studied in white-rot fungi and it has been proved that peroxidases are involved in lignin degradation. However, reports on peroxidase production in brown-rot fungi are few compared with those regarding white-rot fungi.

2-Keto-4-thiomethylbutyric acid (KTBA) is converted to ethylene by one-electron oxidants such as hydroxyl radical, horseradish peroxidase/H_2O_2, ligninase/H_2O_2, tris-(phenanthroline)-iron(III), and ceric ammonium sulfate. Using KTBA, we conducted experiments to determine whether a correlation exists between the degradation of cellulose, lignin, or wood and the extracellular production of one-electron oxidants by brown-rot fungi, and to determine whether the oxidants require H_2O_2 for their activity and are able to catalyze the formation of H_2O_2 in the presence of NADH.

Materials and methods

The brown-rot fungi used for this study are COP: *Coniophora puteana* (Schumacher ex Fr.) IFO 6275, DAD: *Daedalea dickinsii* Yasuda FRI T 4 b, FIH: *Fistulina hepatica* (Schaeff.) (Fr.) IFO 8764, GLS: *Gloeophyllum saepiarium* (Wulf. ex Fr.) Karst. FRI L. 4 e, LAS: *Laetiporus sulphureus* (Bull. ex Fr.) Bond. et Sing. IFO 6497, LEL: *Lentinus lepideus* (Fr.) IFO 8719, LZT: *Gloeophyllum trabeum* (Pers. ex Fr.) Murr. IFO 6268, PHS: *Phaeolus schweinitzii* (Fr.) Pat. IFO 9612, PSU: *Polyporus sulphureus* (Bulliard) Fr. IFO 8833, TYP: *Tyromyces palustris* (Berk. et. Curt.) Murr. FRI 0507 (IFO = Institute for Fermentation, Osaka, Japan; FRI = Forestry and Forest Products Research Institute, Tsukuba, Ibaragi, Japan). They were maintained through periodic transfer on slants as previously described (Takahashi 1976).

The basal agar medium used in this study was prepared as previously described (Enoki et al. 1988).

Wood blocks (2 by 2 by 0.5 cm long) for decay test were prepared from a soft wood, Japanese cedar sapwood (*Cryptomeria japonica* D. Don), and a hard wood, Japanese beech sapwood (*Fagus crenata* Blume). The degrading ability of each fungus was determined using triplicate cultures with two wood blocks as previously described (Enoki et al. 1988).

4-Ethoxy-3-methoxyphenylglycerol-β-guaiacyl ether (I) (Enoki et al. 1980), 1-(3′,4′-dimethoxyphenyl)-1,3-dihydroxy-2-(4″-methoxyphenyl) propane (II) (Enoki and Gold 1982) and dihydroanisoin (III) (Shimada, Gold 1983) were prepared as described previously.

The degrading activity of each fungus was determined using triplicate cultures with 2 mg of each lignin model as previously described (Enoki et al. 1985).

Pure cellulose substrate was prepared from filter papers of 7-cm diameter (Toyo Roshi No 2). The degrading activity of each fungus was determined using triplicate cultures with a filter as previously described (Enoki et al. 1988).

KTBA was exogenously added to cultures containing one substrate – glucose, lignin, cellulose, or wood – as carbon source. Glucose cultures were prepared by adding 200 mg of glucose to 10 ml of basal agar medium in a 50-ml Erlenmeyer flask on a clean bench. Cellulose or lignin cultures were prepared by placing 200 mg of sterilized microcrystalline cellulose powder or Klason lignin isolated from beech wood on 10 ml of basal medium in a 50-ml Erlenmeyer flask on a clean bench. Wood cultures were prepared by placing 200 mg of sterilized sawdust (40 mesh) of Japanese beech wood or of Japanese cedar wood, extracted with distilled water and acetone and dried, on 10 ml of basal agar medium in a 50-ml Erlenmeyer flask. Cultures were inoculated with fungal suspensions, and were incubated at 28 °C in air. At the indicated intervals after inoculation, from the silicon plugs were exchanged to rubber septa and flasks were purged with 100% O_2 or N_2 for 5 min. To each of the cultures, 1 ml of acetate solution (pH 4.5, 20 mM) containing 10^{-5} mol of KTBA was added with a microsyringe through the rubber septa. After additional 24-hrs incubation at 28 °C, 1 ml of gas was removed from the headspace, and the ethylene concentration was monitored directly by gas chromatography at 50 °C in the isothermal mode using a column containing Porapak N. Every ethylene measurement was made in three replicates.

Sterilized sawdust (40 mesh) of Japanese beech wood in 2-g quantities was placed uniformly on the surface of 25 ml of basal agar medium in a 300-ml Erlenmeyer flask. The media were inoculated with small pieces of fungal mat from each test fungus. The cultures were incubated at 28 °C in air. After appropriate periods of incubation, 30 ml of sterilized acetate buffer (5 mM, pH 4.5) was added to the cultures on a clean bench. The mixtures were stirred with a glass rod, shaken for 3 hours at 28 °C in a shaker, and centrifuged at 25,000 G at 4 °C for 20 min. The supernatants collected from 30 flasks incubated with a brown-rot fungus were combined. Acetone (−10 °C) was added to the solution to 70% (V/V). The precipitated protein was centrifuged at 25,000 G at 4 °C for 15 min and resuspended in 100 ml of Na-acetate (pH 4.5, 20 mM). Insoluble material was removed by recentrifugation and the supernatant was dialyzed against 20 mM Na-acetate (pH 4.5). The supernatant was used as an extracellular enzyme. Protein content was determined by the procedure of Lowry et al. (1951).

The basic reaction mixture contained 0.2 mg of extracellular protein, 10^{-5} mol KTBA, and Na-acetate (20 mM, pH 4.5) in a total reaction volume of 3 ml in a 20 ml test tube with rubber septum. Reactions were carried out under 100% O_2 or N_2 at 28 °C. The amount of ethylene gas present in 1 m of headspace in each tube was measured.

Results and discussion

Efficacy of KTBA assay

In initial experiments, we examined the efficacy of the KTBA assay with three oxidants. The results (Table 1) indicate that the following: (1) The generation of

Table 1. Effect of hydroxyl radical scavengers on the production of ethylene gas from KTBA by Fenton's reagent and one-electron oxidants

Addition to reaction mixture[a]	Ethylene production by oxidants					
	$FeSO_4$		$Fe(phen)^{3+}$ [b]		CAS[c]	
	10^{-7} mol	%	10^{-7} mol	%	10^{-7} mol	%
No addition (100% N_2)	0.0	0	4.8	100	4.6	100
10^{-5} mol H_2O_2	7.6	100	–	–	–	–
2×10^{-5} mol Guaiacol	0.0	0	0.0	0	0.0	0
2×10^{-5} mol Guaiacol, 10^{-5} mol H_2O_2	0.3	4	–	–	–	–
2×10^{-5} DMNA[d]	0.0	0	0.0	0	0.0	0
2×10^{-5} mol DMNA, 10^{-5} mol H_2O_2	0.1	1	–	–	–	–

[a] All reaction mixtures contained 2×10^{-3} mol of $FeSO_4$, $Fe(phen)^{3+}$, or CAS, 10^{-5} mol KTBA and distilled water to 3 ml

[b] tris(phenanthroline)iron(III)

[c] Ceric ammonium sulfate = $Ce(SO_4)_2\ 2(NH_3)_2SO_4$

[d] N,N'-dimethyl-4-nitrosoaniline

ethylene from 2-keto-4-thiomethylbutyric acid (KTBA) is not a specific assay for ·OH; (2) etylene is produced by the one-electron oxidation of KTBA; (3) the phenol and the ·OH-scavenging agent inhibit ethylene formation, and (4) the Fenton system is more efficient than the one-electron oxidants, CAS and Fe $(phen)^{+3}$, in ethylene production from KTBA.

Kinetics of wood decay and ethylene production from KTBA

The kinetics of wood-degrading activity and of ethylene production from KTBA were determined (Figs. 1 and 2). No ethylene was detectable in control flasks with no added KTBA. Little ethylene was produced when the assay for ethylene formation was carried out under N_2 instead of O_2. But even in the absence of O_2, the addition of H_2O_2 to the cultures for the assay increased ethylene formation from KTBA (data not shown).

Lentinus lepideus among the brown-rot fungi tested produced the greatest amount of ethylene from exogenously supplied KTBA in cultures containing cedar wood meal and caused the greatest weight loss in the degradation of Japanese cedar wood. *Laetiporus sulphureus, Gloeophyllum trabeum,* and *Tyromyces palustris* showed strong activities in both ethylene production from KTBA and cedar wood degradation. *Daedalea dickinsii, Polyporus sulphureus* and *Phaeolus schweinitzii* produced lower amounts of ethylene and caused lower weight losses in the degradation of the wood than the top four brown-rot fungi. *Coniophora puteana, Gloeophyllum saepiarium* and *Fistulina hepatica* produced little ethylene and almost failed to degrade the wood.

After 3 days, the activity of ethylene formation from KTBA by *L. lepideus* in cultures containing Japanese cedar increased sharply and peaked between days 15 and

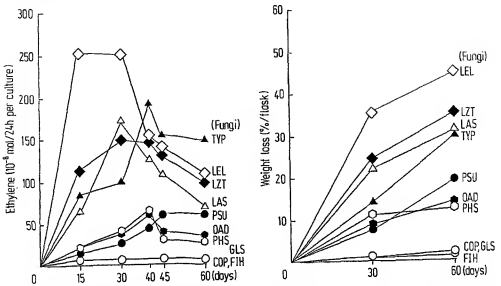

Fig. 1. Ethylene production from KTBA in cultures containing cedar wood and degradation of cedar wood by brown-rot fungi

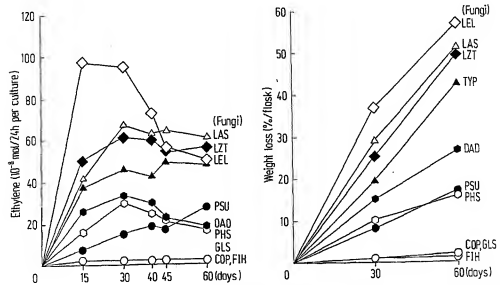

Fig. 2. Ethylene production from KTBA in cultures containing beech wood and degradation of beech wood by brown-rot fungi

30. Then, on the 60th day of incubation, the ethylene production sharply decreased to about one-half of that on the 15th day of incubation. This organism caused a 35% weight loss of cedar wood during the initial 30-day incubation period and a 10% weight loss of the original sound wood during the additional 30-day incubation period.

After 3 days, ethylene production by *G. trabeum* in cedar cultures increased, reached a peak at about 30 days, and then decreased to about two-thirds of that on

the 60th day. This organism caused a 25% weight loss in cedar wood for the first 30 days and a 10% weight loss of the original sound wood over an additional 30 days.

Ethylene production from KTBA by *P. sulphureus* in cedar wood cultures gradually increased, peaked at about 45 days, and then remained almost constant, while the fungus caused a 7% weight loss of cedar wood during the first 30 days and a 13% weight loss of the original sound wood for additional 30 days.

The pattern of ethylene production from KTBA by each of the other brown-rot fungi in cedar wood cultures was correlated with that of cedar wood-degrading activity of each, as shown in Fig. 1. Thus, ethylene production from KTBA was proportional to the degradation activity on wood, and the kinetics of ethylene formation coincided with the appearance of the wood-degrading system. Fig. 2 also indicates that the same correlation exists between ethylene production from KTBA in beech wood cultures and degradation of beech wood by each brown-rot fungus. Therefore, it would appear that extracellular ethylene-producing systems of brown-rot fungi from KTBA are involved in wood degradation and that the assay in cultures of brown-rot fungi grown on wood is a rapid and sensitive measure of wood-degrading activity.

Correlation between degradation in cultures and ethylene production

Extents of degradation of lignin model compounds, of pure cellulose (filter paper), and of beech wood by brown-rot fungi were compared with ethlyene production from KTBA exogenously added to cultures of the fungi containing a corresponding substrate (Table 2).

G. trabeum modified essentially all of the three dimers and produced much more ethylene from KTBA than the other organisms tested in the glucose-containing cultures or the lignin-containing cultures. *L. lepideus* showed a weaker activity against the three dimes than did *G. trabeum* and produced distinct amounts of ethylene from KTBA in glucose- and lignin-containing cultures, considerably less than those produced by *G. trabeum*. Eight of the brown-rot fungi examined showed very poor or no activity against the three dimers and generated little ethylene in either the glucose or lignin cultures. Thus the appearance of the ethylene formation activity was also parallel to that of the degradation activity against the three dimers.

G. trabeum metabolized 80% of the pure cellulose and generated much greater amounts of ethylene from KTBA under the cellulose-culture conditions than did the other fungi. *L. lepideus* caused about one-tenth of the weight loss caused by *G. trabeum* and produced about one-tenth of the amount of ethylene produced by the fungus under the cellulose-culture conditions. The rest of the fungi almost failed to degrade cellulose and generated little ethylene when cultured on cellulose. Thus, ethylene production from KTBA by the brown-rot fungi examined was comparable with degradation activity against cellulose.

The ethylene production from KTBA by the brown-rot fungi examined in cultures containing beech wood was proportional to the degradation ability against beech wood. Three species, *F. hepatica, G. saepiarium,* and *C. puteana,* almost failed to degrade beech wood and produced little ethylene derived from KTBA in cultures containing beech wood meal. The seven other species degraded the beech wood well

Table 2. Ethylene production from KTBA and degradation of various substrates by brown-rot fungi when cultured on each substrate

Fungus	Ethylene production, over time, from substrates				Degradation, over time, substrates							
	20 days		30 days		30 days				120 days		60 days	
	3% Glu.[a]	1% Glu. 2% Lig.[b]	1% Glu. 2% Cel.[c]	1% Glu. 2% Beech[d]	Lignin model				Cellulose[c]		Beech wood	
					I	II	III					
	10^{-8} mol/24 hr		10^{-8} mol/24 h		%	%	%		%	mg/flask	%	mg/flask
G. trabeum	10.5	6.8	31.2	61.4	++++	++++	++++[f]		81	230	49	1,261
L. lepideus	4.2	2.9	3.8	95.5	+++	−	−		7	20	57	1,461
L. sulphureus	0.5	0.2	0.2	67.5	−	+	+		2	6	52	1,379
T. palustris	0.2	0.1	0.1	45.2	−	−	−		3	7	42	1,128
D. dickinsii	0.2	0.1	0.1	32.8	−	−	−		0	0	27	699
P. schweinitzii	0.1	0.1	0.1	30.0	−	−	−		0	0	16	429
P. sulphureus	0.2	0.1	0.1	14.2	−	−	−		0	0	17	463
G. saepiarium	0.4	0.2	0.2	2.0	−	−	−		2	4	2	53
C. puteana	0.2	0.1	0.2	2.1	−	−	−		1	3	2	54
F. hepatica	0.2	0.1	0.1	1.2	−	−	−		0	0	2	49

[a] Glucose, [b] Klason lignin, [c] microcrystalline cellulose, [d] wood meal, [e] filter paper, [f] ++++: 100–80%, +++: 79–60%, ++: 59–40%; +39–20%, −: 19–0%

and generated a large amount of ethylene in the cultures. Five brown-rot fungi, *L. sulphureus*, *T. palustris*, *D. dickinsii*, *Phaeolus schweinitzii*, and *Polyporus sulphureus* almost failed to degrade filter paper and the lignin-model compounds and produced little ethylene from KTBA in cultures containing cellulose or lignin, but they degraded beech wood well and generated a great deal of ethylene derived from KTBA under the beech-wood-culture conditions.

Thus ethylene production from KTBA was related to degradation activities against lignin, cellulose, and wood. Also, a temporal relationship was observed between the appearance of degradation activity against cellulose and that against lignin. Brown-rot fungi necessarily degrade the lignin in wood to a significant extent although they degrade the cellulose in the wood to greater extent (Kirk, Highley 1973; Highley, Murmanis 1985; Highley 1987; Enoki et al. 1988). These facts and the results presented above suggest that brown-rot fungi generate a unique wood-component-degrading system that participates directly or indirectly in the fragmentation of the cellulose as well as of the lignin in wood and oxidizes KTBA to give ethylene.

Extracellular lignin-degrading peroxidases isolated from white-rot fungi catalyzed oxidation of KTBA to give ethylene (Evans et al. 1984; Gold et al. 1984). Lignin model compounds are degraded by Fenton's reagent, but few of the intermediates found in the metabolic pathways of the ligninolytic enzyme of a white-rot fungus or in the intact cultures of the fungus are formed (Kirk et al. 1985). A few brown-rot fungi degrade the compounds (I, II, and III), but none of the products found in the metabolic pathways of white-rot degradation (Enoki et al. 1985). These facts suggest that the ethylene formation system of brown-rot fungi from KTBA is quite different from that of white-rot fungi and may be mediated by ·OH produced in cultures of the brown-rot fungi.

The test fungi varied in their ethylene production depending upon the culture media. *G. trabeum* was able to degrade the three dimers, the pure cellulose, and beech wood in cultures containing glucose as a carbon source. This fungus produced the greatest total amount of the ethylene in the cultures. It generated the least ethylene from KTBA in lignin cultures. *L. lepideus* generated similar amounts of ethylene from KTBA in glucose, lignin, and cellulose cultures, and it degraded cellulose and the dimers to significant extents. This fungus generated about 30 times as much ethylene from KTBA in wood cultures as in the rest of the cultures and degraded beech wood to a great extent. Five species, *L. sulphureus*, *T. palustris*, *D. dickinsii*, *Phaeolus schweinitzii* and *Polypolus sulphureus*, generated little ethylene from KTBA in glucose, lignin, and cellulose cultures. Even so, these fungi generated large amounts of ethylene from KTBA in wood cultures and degraded beech wood to significant extents.

These results indicate that the KTBA-oxidizing system or the wood-component-degrading system of *G. trabeum* effectively is induced by glucose, cellulose, and wood, but not by lignin; that of *L. lepideus* is induced significantly by glucose and most effectively by wood; and *T. palustris*, *L. sulphureus*, *D. dickinsii*, *Phaeolus schweinitzii*, and *Polyporus sulphureus* produce that system only with wood. Thus the production of an extracellular KTBA-oxidizing or wood degrading system by brown-rot fungi is affected differently by carbohydrates present in culture media. In contrast white-rot fungi excrete a KTBA-oxidizing system which is involved in lignin degradation in the presence of glucose (Tanaka et al. 1986).

Table 3. Effect of O_2, N_2 atmospheres, and of H_2O_2, NADH, and ·OH scavengers on the generation of ethylene from KTBA by an extracellular protein isolated from brown-rot fungi

Addition to reaction mixture[a]	Atmosphere	Ethylene production from protein source									
		Laetiporus sulphureus		*Tyromyces palustris*		*Lentinus lepideus*		*Gleophyllum trabeum*		Horseradish peroxidase	
		10^{-8} mol/hr	%	10^{-8} mol/hr	%	10^{-8} mol/hr	%	10^{-8} mol/hr	%	10^{-8} mol/hr	%
None	100% O_2	0.5	3	1.6	6	1.9	7	3.1	11	0.0	0
None	100% N_2	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0
10^{-5} mol H_2O_2	100% N_2	16.2	98	21.0	84	26.1	93	22.0	79	11.3	100
10^{-5} mol NADH	100% O_2	16.5	100	25.0	100	28.0	100	27.8	100	–	–
10^{-5} mol NADH	100% N_2	0.0	0	0.0	0	0.0	0	0.0	0	–	–
10^{-5} mol NADH, 0.3 mg SOD[b]	100% O_2	8.6	52	12.0	48	14.0	50	10.1	46	–	–
10^{-5} mol NADH, 0.3 mg Catalase[c]	100% O_2	0.5	3	0.5	2	1.0	4	0.3	1	–	–
10^{-5} mol NADH, 0.3 mg Denatured catalase	100% O_2	16.4	99	23.2	93	28.3	101	27.6	99	–	–
10^{-5} mol NADH, 2×10^{-5} mol DMNA[d]	100% O_2	2.5	15	5.1	20	5.0	18	5.2	19	–	–
10^{-5} mol NADH, 2×10^{-5} mol Guaiacol	100% O_2	1.3	8	2.8	11	2.8	10	2.0	7	–	–

[a] All reaction mixtures contained 0.2 mg of extracellular protein from one of four fungi or horseradish peroxidase (300 units/mg), 10^{-5} mol KTBA, and acetate buffer (pH 4.5, 40 mM) to 3 ml in a 20 ml test tube; the test tube was purged with 100% O_2 or N_2 for 5 min.

[b] Superoxide dismutase, 2,670 units/mg

[c] 44,200 units/mg

[d] N,N'-dimethyl-4-nitrosoaniline

Involvement of oxidants in ethylene production

Extracellular proteins were isolated from wood-containing cultures of the brown-rot fungi that had caused extensive degradation of wood. Ethylene formation from KTBA by the extracellular proteins and by horseradish peroxidase was examined under various conditions (Table 3). Ethylene production was not observed when the proteins were autoclaved prior to adding KTBA. Nor was ethylene produced from exogenously added KTBA when the reactions were carried out under N_2. But in the presence of H_2O_2, a significant amount of ethylene was produced by the extracellular proteins under anaerobic conditions. In the presence of NADH (or NADPH, data not shown) under 100% O_2, the formation of ethylene from KTBA increased in a range of about 10- and 30-fold. But whether NADH was present or not under the anaerobic conditions, no ethylene was generated. The formation was almost blocked by the presence of catalase. These results suggest that the extracellular proteins of the brown-rot fungi require H_2O_2 to catalyze the oxidation of KTBA to produce ethylene and are capable of catalyzing the oxidation of NADH to produce H_2O_2 in the presence of O_2.

When 0.3 mg (2,670 units/mg) of superoxide dismutase (SOD) was present in the reaction mixtures containing NADH under O_2, the production of ethylene from KTBA was inhibited by about 50%. In presence of SOD (0.3 mg) in the reaction mixtures containing H_2O_2 under N_2, the production of ethylene decreased by only 10 to 20% (data not shown). This observation tends to suggest that $O_2^{\cdot-}$ is involved in the sequence of reactions leading to H_2O_2 production.

Ethylene production by the extracellular proteins was suppressed markedly by the addition of a potent scavenger of ·OH either DMNA or guaiacol, to the reaction mixtures. Horseradish peroxidase also oxidized and converted KTBA into ethylene.

The phenol-oxidase activity of the extracellular proteins, horseradish peroxidase, Fenton's reagent, and one-electron oxidant was determined with guaiacol and with dichlorophenol (Table 4). Neither the extracellular proteins nor Fenton's reagent polymerized the phenols to colored products. In contrast, oxidant CAS and horseradish peroxidase/H_2O_2 polymerized the phenols to colored products as expected. The oxidation activity of each extracellular protein against KTBA was higher than that of horseradish peroxidase in the same concentration (mg/ml) (Table 3). As the ethylene formation by the extracellular proteins was markedly inhibited by guaiacol, the oxidation systems must have acted on the phenol.

The hydroxyl radical mediates the hydroxylation and decarboxylation of benzoic acids (Butler, Mason 1961; Matthews and Sangster 1966), but as shown here, does not cause phenol oxidase reactions. Superoxide weakens the bonding between the fibrillar components of cellulose and markedly decreases the degree of polymerization (DP) (Thompson, Corbett 1985). A fairly dilute hydrogen peroxide solution (1 mM) depolymerizes cellulose in the absence of metallic catalysts (Miller 1986). These facts and the results presented here suggest that partially reduced oxygen may play a role in KTBA oxidation to give ethylene or in wood degradation in the cultures of brown-rot fungi.

The results and the hypothesis on the mechanism of wood degradation or KTBA oxidation by brown-rot fungi presented above are summarized and illustrated in Fig. 3.

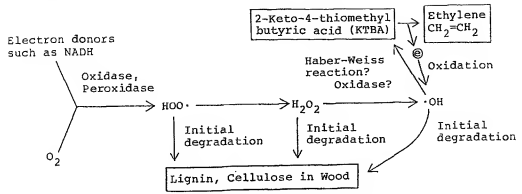

Fig. 3. Scheme of proposed initial degradation mechanism of the cellulose and the lignin in wood by brown-rot fungi

Table 4. Phenol-oxidase activity of crude extracellular proteins isolated from 30-day-old cultures containing beech wood, Fenton's reagent, and one-electron oxidant

Protein source	Addition to reaction mixture[a]	Absorbance[b]	
		with guaiacol (5×10^{-4} M)	with aminoantipyrine (10^{-4} M) and 2,4-dichlorophenol (10^{-3} M)
		Δ_{420}	Δ_{510}
Selected fungi[c]	0.2 mg	0.0	0.0
Horseradish peroxidase	0.004 mg	1.5	2.5
$FeSO_4$	10^{-3} M	0.0	0.0
CAS[d]	10^{-3} M	1.4	2.0

[a] All reaction mixtures contained 10^{-3} M H_2O_2 and 3 ml of acetate buffer (pH 4.5, 40 mM).
[b] Change in absorbance was determined after 120-min incubation at 28 °C.
[c] *Laetiporus sulphureus, Tyromyces palustris, Lentinus lepideus,* or *Gloeophyllum trabeum.*
[d] Ceric ammonium sulfate

Experiments for the detection of oxygen radicals produced extracellularly in the cultures of brown-rot fungi are being carried out. Further, the purification and characterization of extracellular KTBA-oxidizing and H_2O_2-producing enzymes of brown-rot fungi are now in progress.

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